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Original Article

Phytochemical Screening, Antibacterial Activity, and Mode of Action on *Morus nigra*

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ABSTRACT

Introduction: The *Morus nigra* (*Moraceae*) known as black mulberry is a well-grown plant in Lembang, Indonesia. Previous studies showed that black mulberry fruit has activity as antibacterial and antioxidant. The aims of this study were to evaluate the antibacterial effect of ethanol extract from stem bark, fruit, and leaves of *Morus nigra*, MIC and morphological changes of the most active extract against *Staphylococcus epidermidis* and *Propionibacterium acnes*. **Methods:** Agar diffusion and microdilution assays were used to evaluate the antibacterial effect of the extract by measuring the zone of inhibition and the minimum inhibitory concentration (MIC) of the extract against the test bacteria. Scanning electron microscopy (SEM) was performed to evaluate the morphological changes induced by the extract in cellular membrane of the bacteria. Protein and ion leakage from the bacterial cells induced by the extract were also evaluated. **Results:** The antibacterial activity showed the most active extract was an ethanol extract of the stem bark against *S. epidermidis* and *P. acnes* with MIC value 4 mg/ml and 2 mg/ml, respectively. The extract also induced protein and ion leakage in test bacterial cells. SEM findings revealed that the extract induced potential cellular membrane damage in 4xMIC. **Conclusion:** The antibacterial activity was related to the damaged of bacterial cell wall.

Key words: *Morus nigra*, *Staphylococcus epidermidis*, *Propionibacterium acnes*, Cellular membrane damage.

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INTRODUCTION

In a few decades, many antibiotics were found to be resistance.[1] This has led to search for new, safe and effective antibacterial agents for natural plants.[2] Indonesia have so many biodiversity, one of the plant that belong to family Moraceae, *Morus nigra* or known as black mulberry, a plant native to China and nowdays cultivated in several countries, including Indonesia. The fruit is purplish red and has sweet flavor. There are several compounds that have been studied on *Morus nigra* L, such as fenol, flavonoid (kuwanon E and kuwanon U, and morusin), chalcone, 1-deoxynojirimycin.[3,4,5,6]

Empirically in China, black mulberry plants are often used to decrease blood pressure, anti-hyperglycemic, diuretic, antioxidant, anthelmintic, anti-parasitic and antibacterial.[6,7,8,9] Black mulberry was known could inhibit bacterial i.e. *Bacillus subtilis, Micrococus flavus, Streptococcus faecal, Staphylococcus aureus, Eschericia coli*, and *Pseudomonas aeruginosa*.[4,10] Previous studies showed that the ethanol extract of *Morus nigra* fruit has activity against *Propionibacterium acnes* (MIC 2.5 %) and *Staphylococcus epidermidis* (MIC 2.5 %).[11] Acne vulgaris is the most common skin desease. *Staphylococcus aureus, Staphylococcus epidermidis*, and *Propionibacterium acnes* were proliferate during puberty and can developed acne.[1,12]

The Antibacterial method should be posibble for extracts, fractions and isolates. Its method should be simple, rapid, reproducible, and inexpensive.[13] One of the most commonly used for antibacterial activity methods are diffusion and dilution method. This research will reported the antibacterial activity and mode of action on *Morus nigra*.

MATERIALS AND METHODS

General experimental apparatus

The tools used are: maceration equipment, Incubator (Jenaco®), autoclave (My Life®), spectrophotometer UV-Vis (*Shimadzu UV-VIS 1800*), Atomic Absorption Spectroscopy (*Shimadzu*), *Scanning Electron Microscope* (SEM) (JEOL JSM-5310LV®), *ion coater* (Ion Coater IB2®).

MATERIALS

Black Mulberry (*Morus nigra*) obtained from the mulberry Plantation field in Bandung, Indonesia and authenticated in Herbarium Bandungense, Bandung Institute of Technology, Indonesia. The samples were dried in drying cabinet (40-50 °C), and cut into fine pieces.

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Determination of Antibacterial activity

Microorganisms used

Staphylococcus epidermidis (*S. epidermidis*) and *Propionibacterium acnes* (*P. acnes*) were obtained from the collection of Microbiology Laboratory, Faculty of Pharmacy, Universitas Padjadjaran.

Culture media Bacterial inoculum

S. epidermidis and *P. acnes* were maintained on Muller Hinton Agar (MHA) (Merck) Petri dish sterile for 24 h at 37°C. The turbidity of the resulting suspensions was diluted with sodium chloride 0.9 % w/v to obtain a transmittance of 25.0 % at 580 nm. The percentage was compared to McFarland turbidity standard using UV Spectrophotometry (Shimadzu®). The level of turbidity is equivalent to approximately 3.0 x 10^8 CFU/ml.

Phytochemical Analysis

To determine the content of secondary metabolites contained in black mulberry (*Morus nigra*) is carried out phytochemical screening is test of alkaloids, flavonoids, tannins, polyphenols, monoterpenes, sesquiterpenes, steroids, triterpenoids, quinones, saponins.[14]

Extraction

Dried black mulberry stem bark, fruit, and leaves (@200 g; 2 litre) was extracted by maceration method using ethanol as solvent for 3x24 h, then concentrated to dryness under vacuum at temperature 45°C by using rotary evaporator (IKA®).

Antibacterial Activity

The antibacterial activity was performed using Agar Diffusion method on ethanol extract of stem bark, fruit, and leaves of black mulberry. 150 µl bacterial suspension was added into 15 ml of medium and homogenized, then incubated at 37 °C for 24 h. The extract was dissolved in 1% v/v DMSO to obtain four concentrations: 50; 25; 12.5 and 6.25% w/v. Clindamycin (50µl) was used as positive control and 1% v/v of Dimethyl sulfoxide (DMSO) solution was used as negative control. The antibacterial activity was experimented in triplicate, and the mean value was also determined.[12]

Minimum inhibitory concentration (MIC) evaluation

MIC was determined by a micro-dilution method using a microplate. 100 µl MHB media added into 100 µl of extract. Furthermore, each well was added 10 µl of the bacterial suspension was adjusted to McFarland turbidity standards was equivalent to approximately 3.0 x 10^8 CFU/ml. Furthermore, the microplate was covered with a plastic wrap and then incubated at 37°C for 24 h.[15]

Cell Morphological Observation

Clear zone derived from the treatment in the antibacterial test soaked with a solution of 2% glutaraldehyde overnight. Test solution is centrifuged and the supernatant discarded. The residue was added to a solution of 2% tannic acid, then soaked a few h. Test solution was centrifuged back and disposed of fixative solution was then added chocodylate buffer, and soaked for 20 min. Test solution centrifuged and the supernatant is separated, then added 1% osmium tetraoxide and soaked for 1 hour. The test solution was centrifuged and the supernatant discarded and soak together 50% alcohol for 20 min. The residue is dried in a row with alcohol 70%, 80%, 95% and absolute alcohol for 20 min each. Samples were suspended with the addition of buthanol and soaked 20 min, then the suspension placed on the cover slip, dried with a freeze dryer and then coated with gold and observed using *Scanning Electron Microscopy* (SEM) (JEOL JSM-5310LV®).[16,17]

Analysis of Protein and Nucleic Acid Leakage

The bacterial suspension that has been grown for 24 h in 10 mL Mueler Hinton Borth (MHB) medium was taken and centrifuged at 3500 RPM for 20 m. The filtrate was discarded and the pellets in the tube were washed with phosphate buffer (pH 7.0) solution for 2 times, then suspended in 10 ml of phosphate buffer (pH 7.0) solution and shaken for 24 h. Furthermore, the black mulberry stem bark extract was added with concentration of 1xMIC, 2xMIC, and control into suspension. Suspension is centrifuged at 3500 RPM for 15 min, and then filtered in order to separate the supernatant and cells. Supernatant fluids were taken and measured their absorbance at wavelengths of 260 nm and 280 nm using UV/VIS spectrophotometry (Shimadzu UV-VIS 1800).

Analysis of Ion Leakage

Ion leakage analysis is performed on prepared bacterial pellets such as on leakage of proteins and nucleic acids. Leakage of cells is expressed by measurements of ions (Ca^{2+} and K^+) present in test bacteria after contact with 4xMIC concentrations. The leakage of Ca^{2+} and K^+ ions is measured using AAS (Shimadzu).[18]

RESULTS AND DISCUSSION

The phytochemical screening was done to the crude drug, ethanol extract of stem bark, fruit, and leaves Table 1. The essential oil of monoterpene and sesquiterpen were absence on leave. *Morus nigra* belongs to Genus Morus known rich in phenolic such as kuwanon E and kuwanon U, chalcone, morusin, moracin, anthocyanin, vanillic acid hexoside, 5-p-O-coumaroylquinic acid, 4,5-di-O-caffeoylquinic acid, luteolin hexoside, luteolin rutinoside, and quercetin rhamnosyl dihexoside.[19,20,21]

Among three extracts that were investigated for antibacterial activity, two extracts including stem bark and fruit ethanol extract had an inhibitory effect against *S. epidermidis* and *P. acnes,* but had moderate activity on leaves ethanol extract of black mulberry (Graphic 1). Diffusion method was chosen because this method was simple, rapid, reproducible, and inexpensive.[13] This diffusion method is not recommended for natural water-insoluble antimicrobial compounds, such as steroid, terpenoid and essential oil. The hydrophobic nature of the natural compound prevents uniform diffusion through the agar medium.[22] In this study, the substances used to suspense the extract were DMSO 1% as solvent. After being suspended, the extracts could diffused in the media, and inhibited the growth of bacterial.

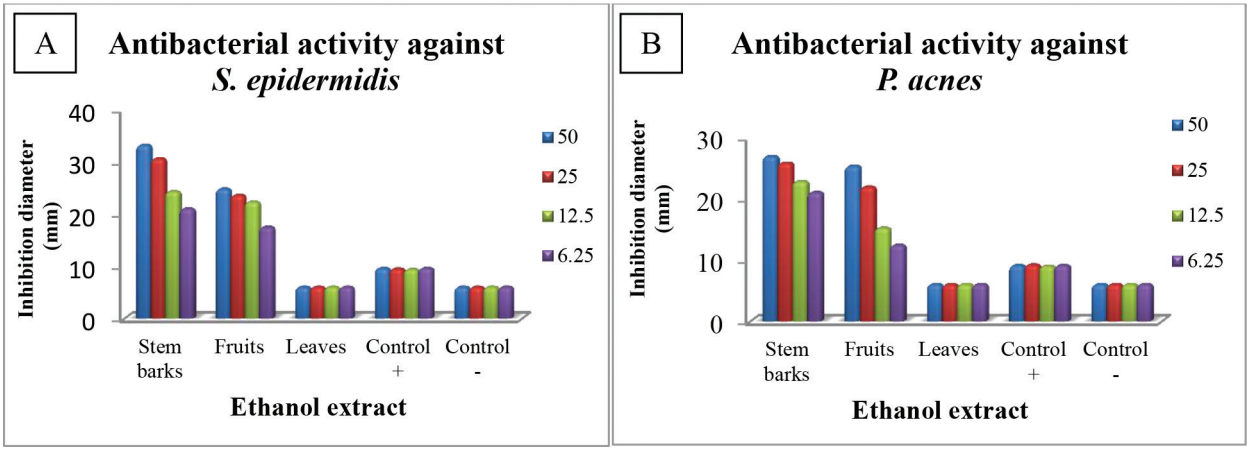
The minimum inhibitory was continued on stem bark ethanol extract due to the stable inhibitory Figure 1 against *S. epidermidis* and *P. acnes* with MIC value 4 mg/ml and 2 mg/ml. To observe a morphological alteration after the cell was treated with the most active extract, stem bark ethanol extract, against *P. acnes,* a bacterial specific caused acnes by using scanning electron microscope. The bacterial *P. acnes* cells treated with 4xMIC stem bark ethanol extracts were compared with untreated cells, the treated cells appeared to be shrinking and there was a degradation of the cellular membrane as shown in Figure 2.

The cellular membrane of the *Propionibacterium sp* contains mucopeptide components such as glutamic acid, alanine, diaminopimelic acid, muramic acid and glucosamine, also minerals.[23] The other research has studied about the cellular membrane of spesies *Propionibacterium acnes*, explained that these cellular membrane contains acidic polysaccharide consisting of neutral sugars and amino sugars, without phosphorous.[24] These may explained the phenolic such as flavonoid, chalcone, stilbene compounds with many hydroxyl groups could diffuse through the microbial membrane, and also the hydroxyl may attached to an aromatic ring was required for antibacterial activity.[25] Thus, antibacterial activity and membrane interference supports the theory that flavonoids also may demonstrate antibacterial activity by reducing membrane fluidity of

Table 1: The Phytochemical Screening of Crude Drugs, Ethanol Extract And Stem Barks Fraction

Compound group	Crude drugs			Ethanol extract		
	Stem bark	Fruit	Leaves	Stem bark	Fruit	Leaves
Alkaloid	-	-	-	-	-	-
Flavonoid	+	+	+	+	+	+
Tannin	+	+	+	+	+	+
Monoterpene	+	+	-	+	+	+
Sesquiterpene	+	+	-	+	+	-
Steroid	-	-	-	-	-	-
Triterpenoid	-	-	-	-	-	-
Quinone	+	+	+	+	+	+
Saponin	+	-	-	-	-	-
Phenolic	+	+	-	+	+	-

Note: + = detected, - = not detected



Graphic 1: Inhibition zone (diameter in mm) of stem barks, fruits, leaves of *Morus nigra* against (A) *S. epidermidis*, and (B) *P. acnes*.
Note: Perforator: 6 mm, Control (-):1% v/v DMSO as solvent, Control (+): Clindamycin 50µg/ml

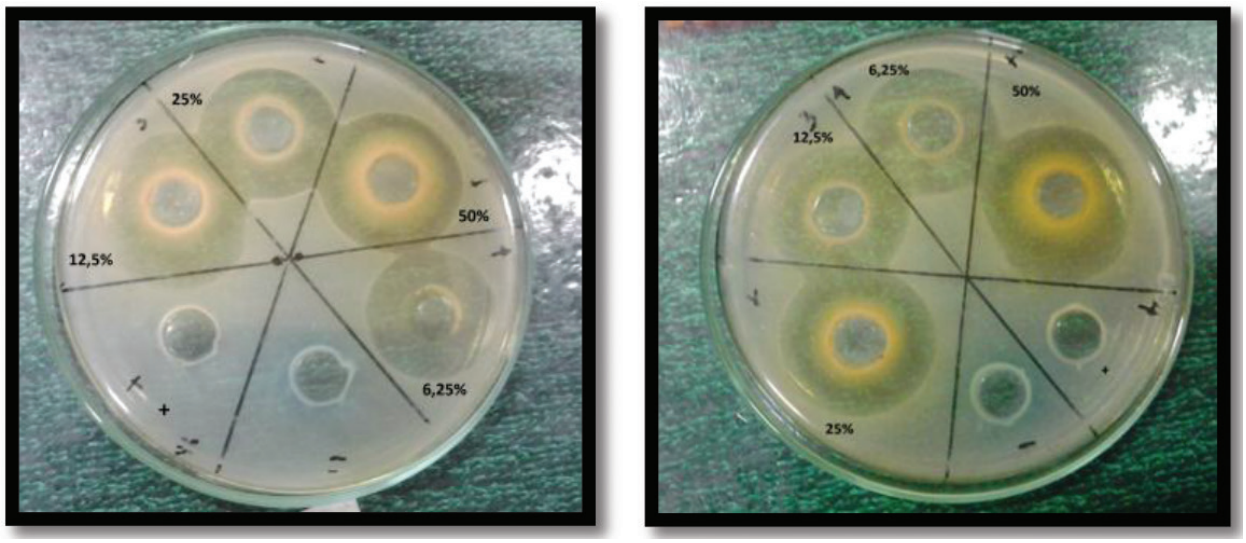


Figure 1: Inhibition zone of stem barks against (I) *S. epidermidis*, and (II) *P. acnes*.

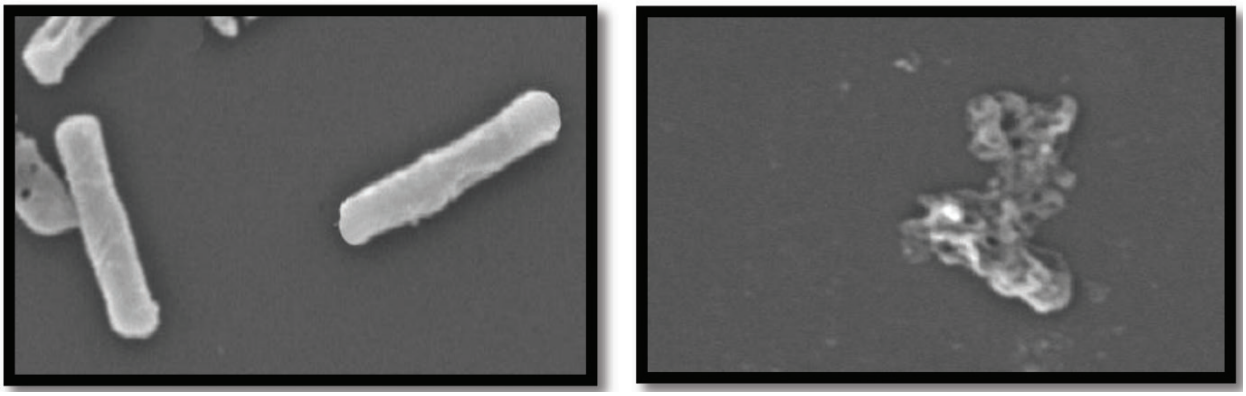
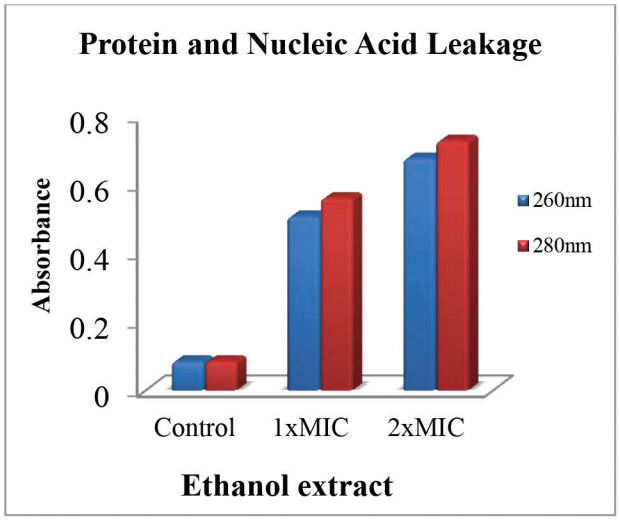
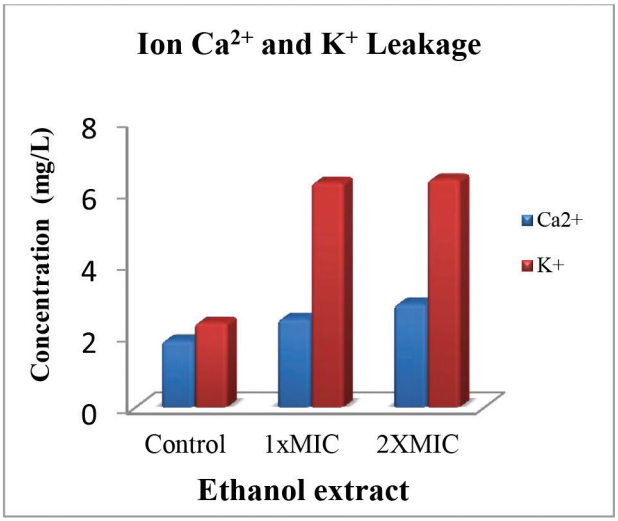


Figure 2: *Propionibacterium acnes morphological cellular* in scale 7500x (a) untreated, (b) bacteria treated with stem bark ethanol extract 4 x MIC.



Graphic 2: Nucleic acid (260 nm) and protein (280 nm) leakage with 1xMIC and 2xMIC of black mulberry stem bark ethanol extract against *P. acnes*.



Graphic 3: Absorbance of ion calcium (Ca^{2+}) and Kalium (K^{+}) with 1xMIC and 2x MIC of black mulberry stem bark ethanol extract against *P. acnes*.

bacterial cells and causing cell fluid imbalance (Graphic 2 and Graphic 3).[26] According Khalid researched, the total phenolic contents of fresh mulberry juice about 2050 μg/g as gallic acid equivalent (GAE) (Khalid, Fawad, and Ahmed 2011)the fresh juice of black mulberry (*Morus nigra)*. The black mulberry especially fruit and stem bark has excellent activities as antimicrobial agent.[29]

CONCLUSION

The stem bark and fruit of *Morus nigra* or black mulberry were found to be potentially as the source of herbal cosmetic such as in acne treatment. This study showed that the mode of action of the stem bark ethanol extract was related to the damaged of bacterial cell wall.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest, financial, or otherwise regarding the publication of this paper.

ABBREVIATIONS USED

MIC: Minimal Inhibitory Concentration; **MHA:** Mueller Hinton Agar; **MHB:** Mueller Hinton Broth; **CFU:** Colony Forming Units, **DMSO:** Dimethyl Sulfoxide.

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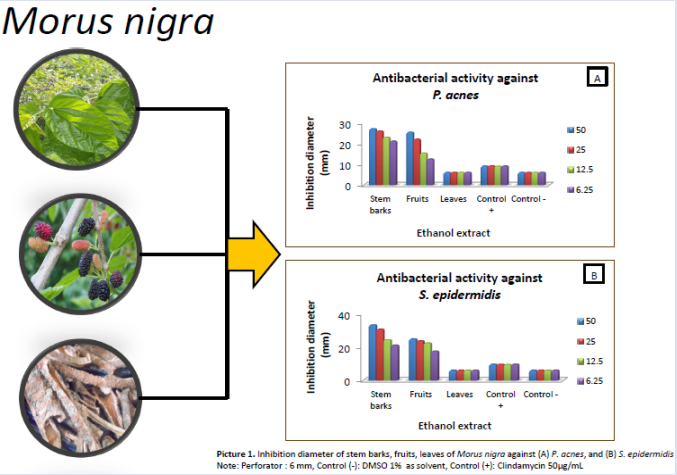
GRAPHICAL ABSTRACT



Picture 1. Inhibition diameter of stem barks, fruits, leaves of *Morus nigra* against (A) *P. acnes*, and (B) *S. epidermidis*
Note: Perforator : 6 mm, Control (-): DMSO 1% as solvent, Control (+): Clindamycin 50µg/mL

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